Purification and Some Properties of an Extracellular Amylase from a Moderate Halophile, *Micrococcus halobius*

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A moderate halophile, *Micrococcus halobius* ATCC 21727, produced an extracellular dextrinogenic amylase when cultivated in media containing 1 to 3 M NaCl. The amylase was purified from the culture filtrate to an electrophoretically homogenous state by glycogen-complex formation, diethylaminoethyl-cellulose chromatography, and Bio-Gel P-200 gel filtration. The enzyme had maximal activity at pH 6 to 7 in 0.25 M NaCl or 0.75 M KCl at 50 to 55°C. The activity was lost by dialysis against distilled water. Molecular weight was estimated to be 89,000 by sodium dodecyl sulfate-gel electrophoresis. The action pattern on amylose, soluble starch, and glycogen showed that the products were maltose, maltotriose, and maltotetraose, with lesser amount of glucose.

In a previous paper (10), one of the authors reported that a moderate halophile, *Micrococcus halobius* (12), produced an extracellular dextrinogenic amylase when cultivated in media containing 1 to 3 M NaCl. Subsequently, Onishi and Hidaka (11) reported that another moderate halophile, *Acinetobacter* sp., produced dextrinogenic amylase when cultivated in media containing 1 to 2 M NaCl or 1 M KCl. Two kinds of *Acinetobacter* amylase, amylases I and II, were purified from the culture filtrate to an electrophoretically homogenous state, and the characteristics of the amylases were demonstrated (11). Good and Hartman (5) investigated properties of the amylase from an extreme halophile, *Halobacterium halobium*, although the enzyme was not purified. As for halophilic amylase, only *Acinetobacter* amylases have so far been purified.

It is interesting to compare the characteristics of the purified enzyme preparations. The present paper describes purification and some properties of the *M. halobius* amylase.

MATERIALS AND METHODS

Bacterial strain. *M. halobius* ATCC 21727, isolated from unrefined solar salt by Onishi and Kamekura (12), was used in this study.

Amylase production for enzyme purification. A modified Sehgal and Gibbons complex medium (18; SGC) of the following composition was used for amylase production for enzyme purification: 0.75% vitamin-free Casamino Acids (Difco), 1.0% yeast extract (Difco), 0.3% sodium citrate, 0.2% KCl, 2% MgSO$_4$· 7H$_2$O, 5.85% (1 M) NaCl, 2.3 mg of FeCl$_2$·nH$_2$O per 100 ml, and 1.0% soluble starch, pH 6.4 to 6.6. Inocula (0.5 ml) of a 2-day-old culture grown in the same

medium were added to 500-ml shaking flasks containing 80 ml of the medium. The flasks were shaken at 30°C on a reciprocal shaker operating at 140 rpm with a stroke of 7.5 cm for 2 days.

Amylase assay. Dextrinogenic amylase activity was assayed by using soluble starch as a substrate by the modified method of Fuwa (4): 2 ml of 0.5% soluble starch in 25 mM tris(hydroxymethyl)aminomethane(Tris)-hydrochloride buffer (pH 7.0) containing 2 M NaCl was mixed with 1 ml of enzyme solution. After incubation at 50°C for 10 min, a 0.2-ml portion of the reaction mixture was added to 5 ml of 0.167 mM I$_2$-KI solution. The optical density at 700 nm was measured in a spectrophotometer. Hydrolysis of 0.1 mg of soluble starch in 1 min was defined as 1 U of enzyme activity. In some experiments, changes in NaCl or KCl concentrations, reaction pH and temperatures of enzyme assay were made.

Determination of protein. Protein concentrations during most stages of enzyme purification were determined by the Folin phenol method (7). Bovine serum albumin was used as the standard. The protein concentration of each fraction during column chromatography was estimated by observing the absorbance at 280 nm.

Determination of sugar. Reducing and total sugars were determined by the methods of Somogyi-Nelson (19) and of phenolsulfuric acid (2), respectively.

Disc electrophoresis. Electrophoresis in polyacrylamide gel was carried out by the method of Davis (1) with the modification that all gels and buffer contained 5 mM CaCl$_2$. Standard 7.5% polyacrylamide gels (pH 8.0) were used. Samples were loaded on the column (0.5 by 8 cm) in glycerol and subjected to electrophoresis for 3 to 4 h at 4 mA/gel column. Sodium dodecyl sulfate-polyacrylamide disc gel electrophoresis was performed by the method of Weber and Osborn (23).

Action pattern on substrates. Multiple-ascent

paper chromatography was performed for the analysis of the products of the enzyme digestion as described by Robyt and French (15, 16). The substrates used were amylose, soluble starch, and glycogen, and their concentrations were 0.5, 1, and 1%, respectively. The reactions were done at pH 6.5 and 50°C. Samples were taken at various times, and the reaction was stopped by boiling. After four ascents on Whatman no. 3MM paper with the solvent of *n*-butanol–pyridine–water (6:4:3[vol/vol/vol]), the chromatograms were developed with the silver nitrate dip procedure of Robyt and French (15, 16).

RESULTS

Enzyme purification. Procedures developed for purification of amylase are detailed in the following steps.

Step 1. Cell-free filtrate. The 2-day-old culture broth was subjected to centrifugation to remove cells, yielding about 8.27 liters of clear solution with a protein concentration of 2.5 mg/ml and a specific activity of 21.2 U/mg of protein.

Step 2. Formation of the glycogen-amylase complex. The method of Loyter and Schramm (8) was applied. The cell-free filtrate was cooled overnight, and cold ethanol was added to it with stirring to 40% final concentration. After removing the resulting precipitate, 1/10 volume of cold 2% glycogen solution was added to the clear supernatant with stirring to form the glycogen-amylase complex. The complex was collected by centrifugation and dissolved into 600 ml of 0.05 M Tris-hydrochloride buffer (pH 7.0) containing 50 mM CaCl$_2$.

Step 3. Diethylaminoethyl-cellulose column chromatography. The complex solution was incubated for 3 h at 30°C and dialyzed against Tris buffer containing 50 mM CaCl$_2$ to allow for complete dissociation and amylolysis of glycogen. The dialysate (750 ml) was passed through a diethylaminoethyl-cellulose column (1.8 by 45 cm) equilibrated with 50 mM Tris-hydrochloride buffer (pH 7.0) containing 50 mM CaCl$_2$ (Fig. 1). The amylase was absorbed into the column. A linear gradient elution was carried out in the cold in 50 mM Tris-hydrochloride buffer (pH 7.0) containing 50 mM CaCl$_2$ between 450 ml each of 0 and 0.5 M NaCl. Fractions of 10 g were collected, and the enzyme was eluted between 0.30 and 0.45 ionic strength.

Step 4. Bio-Gel P-200 column chromatography. The active fraction (total 145 ml, nos. 41 to 54 in Fig. 1) was concentrated to 1.2 ml by Diaflo membrane and fractionated on a Bio-Gel P-200 column (1.8 by 100 cm) after the column had been equilibrated by passing 1 liter of 0.05 M Tris-hydrochloride buffer (pH 7.0) containing 50 mM CaCl$_2$ and 1 M NaCl through it; fractions of 3 g were collected. Figure 2 shows that in the
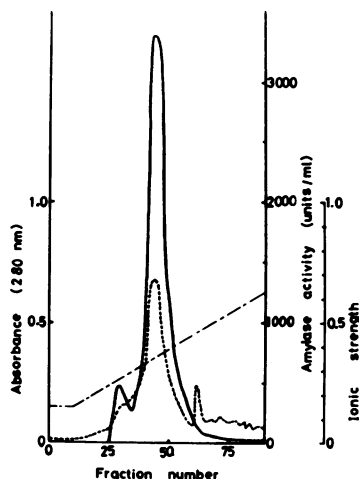

FIG. 1. *Elution pattern of diethylaminoethyl-cellulose column chromatograph of the Micrococcus amylase. Diethylaminoethyl-cellulose was hydrated, packed in a column (1.8 by 45 cm), and equilibrated with 50 mM Tris-hydrochloride buffer (pH 7.0) containing 50mM CaCl$_2$. The dialyzed glycogen-complex solution was loaded, the enzyme was adsorbed well in the column, and for elution a linear gradient of 450 ml each of 0 and 0.5 M NaCl in the buffer was used. The elution flow rate was 38 ml/h, and 10-g fractions were collected. Symbols: ——, amylase activity; - - - - -, protein; –·–·–·–·–, NaCl.*

peak fractions of eluted enzyme there was a coincidence of protein concentration and amylase activity.

The purification steps, summarized in Table 1, disclose 474-fold purification over the culture filtrate with a 47% recovery of activity.

The homogeneity of the purified enzyme preparation was examined by polyacrylamide disc gel electrophoresis and gave one band on the gel (data not shown).

Enzyme characteristics. The effects of pH on the enzyme activities at 50°C are shown in Fig. 3. The optimum pH for the enzyme was 6.5. To determine the pH stability, portions of the enzyme preparation were held at 50°C for 30 min in 33 mM acetate buffer (pH 4.0 to 6.5), 33 mM Tris-hydrochloride buffer (pH 7.0 to 9.0) or 33 mM glycine-NaOH buffer (pH 10.0) containing 1 M NaCl and 50 mM CaCl$_2$, and residual activity was determined by the standard assay. The enzyme activity was stable at pH 6.5 to 7.5.

The enzyme activity was assayed at various temperatures at pH 7.0 for a period of 10 min (Fig. 4). The optimum temperature was around 50 to 55°C. At pH 7.0, the enzyme was stable to 30 min of incubation at 30°C, and losses in activity of 20% (in 50 mM CaCl$_2$) and 40% (in 0.19 mM CaCl$_2$) were observed as temperature
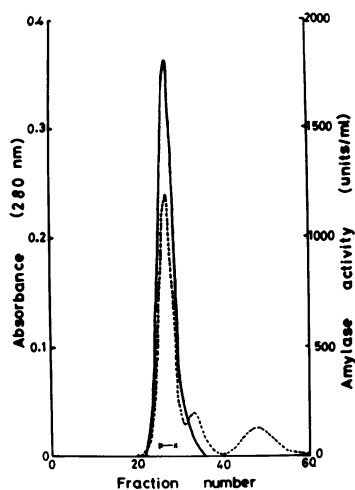
FIG. 2. Elution pattern of the amylase from Bio-Gel P-200 column. Bio-Gel P-200 was hydrated, packed in a column (1.8 by 100 cm), and equilibrated with 50 mM Tris-hydrochloride buffer (pH 7.0) containing 50 mM CaCl$_2$ and 1 M NaCl. Elution was carried out at a flow rate of 8 ml/h with the same buffer used for equilibration, and 3-g fractions were collected. The amylase assay and protein determination were carried out by using a 1/10 dilution of each fraction. Symbols: ——, amylase activity; -----, protein; |←—→|, fractions collected for examination of homogeneity.

was increased to 50°C. At 60°C, 60% of the activity was lost regardless of the presence of CaCl$_2$.

The molecular weight of the purified amylase, as determined by sodium dodecyl sulfate-disc gel electrophoresis, was 89,000 (Fig. 5).

The enzyme required 0.25 M NaCl or 0.75 M KCl for maximal activity at pH 7.0 in 33 mM Tris-hydrochloride buffer. When 33 mM phosphate buffer was used instead of Tris-hydrochloride buffer, the salt requirements were enhanced (Fig. 6).

When the enzyme was dialyzed against distilled water or 0.01 M ethylenediaminetetraacetic acid (pH 6.5), the activity was completely lost within 24 and 8 h, respectively. The enzyme was fairly stable in 0.05 M CaCl$_2$ but rather unstable in 2 M NaCl or KCl, showing 63 to 64% loss of activity after 24 h. Attempts to obtain reactivation of the inactivated enzyme by dialysis against 50 mM CaCl$_2$ were unsuccessful.
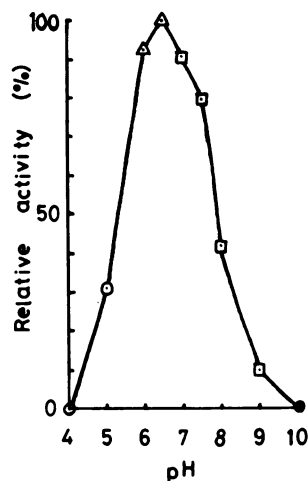

FIG. 3. Effect of pH on the Micrococcus amylase activity. The activity was measured in the presence of 1 M NaCl at 50°C for 10 min. The enzyme activity assayed at pH 6.5 was taken as 100%. The buffers used were 33 mM acetate buffer (○, pH 4.0 to 5.0), 33 mM phosphate buffer (△, pH 6.0 to 6.5), 33 mM Tris-hydrochloride (□, pH 7.0 to 9.0), and 33 mM glycine-NaOH (●, pH 10.0).

The enzyme transformed about 35% of the starch to reducing sugar (calculated as glucose), and the color of the iodine reaction disappeared at 19% hydrolysis (data not shown).

The action pattern of the enzyme on amylose, soluble starch, and glycogen was examined (Fig. 7). Early reaction products from amylose, soluble starch, and glycogen digests included maltose through maltotetraose; little glucose was observed. As the reaction proceeded, products included predominantly maltose, maltotriose, and maltotetraose, with lesser amounts of glucose. In various studies (3, 21, 24) on the correlation of carbohydrate structure with paper chromatogram mobility, a plot of log (R_f/1-R_f) against the degree of polymerization of oligosaccharides was a straight line. When R_f values of glucose, maltose, maltotriose, and an unknown spot were subjected to this treatment, a straight line was formed. An unknown spot appears to be maltotetraose when estimated in this manner.

DISCUSSION

It is known that most of bacterial liquefying α-amylases are well absorbed to starch and

TABLE 1. Purification scheme for M. halobius amylase

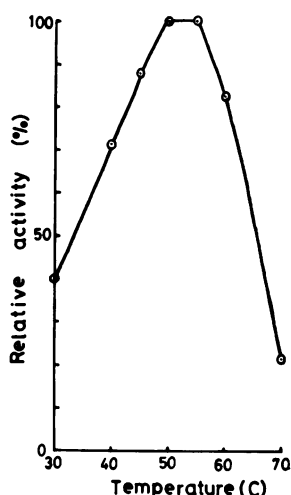| Purification step	Vol (ml)	Total protein (mg)	Total activity (U)	Sp act (U/mg of protein)	Yield (%)	Purification (fold)
Culture filtrate	8,270	20,657	438,310	21.2	100	1
Dialysate of glycogen complex	750	124.5	410,244	3,295.1	93.6	155.4
Diethylaminoethyl-cellulose	145	33.93	294,754	8,687.1	67.2	409.8
Bio-Gel P-200	17	20.42	205,348	10,055.0	46.8	474.3

FIG. 4. *Effect of temperature on the Micrococcus amylase activity. Amylase activities at various temperatures were measured in 33 mM Tris-hydrochloride buffer (pH 7.0) containing 1 M NaCl for 10 min. The enzyme activity at 55°C was taken as 100%.*
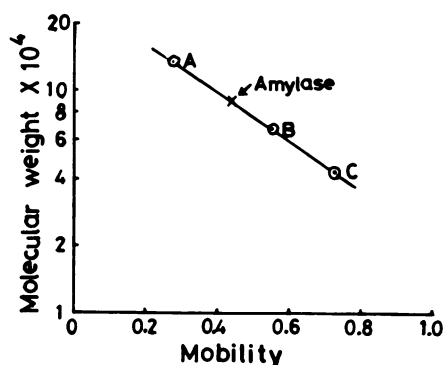

FIG. 5. *Molecular weight determination of the purified Micrococcus amylase by sodium dodecyl sulfate-acrylamide disc gel electrophoresis using 5% gel. Molecular weights of the standard proteins were as follows: (A) bovine serum albumin dimer, 136,000; (B) bovine serum albumin monomer, 68,000; (C) ovalbumin, 43,000.*

eluted by buffers of pH value above 8, but not by water (20). However, the *M. halobius* amylase showed little adsorption to starch and enough complex formation with glycogen. The fact was effectively applied in the enzyme purification. For purification of the *Micrococcus* amylase, diethylaminoethyl-Sephadex A-50 column chromatography and Sephadex G-200 gel filtration were not successfully applied, unlike the *Acinetobacter* amylase. Instead, diethylaminoethyl-cellulose column chromatography and Bio-Gel P-200 gel filtration were used.

The molecular weight of many bacterial α-amylase is about 50,000 (6, 17), and those of the

halophilic *Acinetobacter* amylase I and II are 55,000 and 65,000, respectively (11). The molecular weight of the purified *Micrococcus* amylase, determined by sodium dodecyl sulfate-disc gel electrophoresis, was 89,000 (Fig. 5). The result resembles that of the *Bacteroides amylophilus* α-amylase (92,000) (9), which suggested the possible existence of a dimer. It is known that many proteins (13), including *Bacillus subtilis* α-amylase (14), form micellar complexes with sodium dodecyl sulfate and do not undergo dissociation (13).

The characteristic blue-value–reducing-value curve of the purified amylase identified it as being an α-type amylase. A major portion of the curve corresponded to that of *B. subtilis* α-amylase.

Degradation products in the *M. halobius* amylase digests (Fig. 7) were similar to those of many other α-amylases previously examined (17).

The salt concentration required by the purified enzyme for the maximal activity shifted to lower levels of 0.25 M NaCl or 0.75 M KCl at pH 7.0, compared with 1.4 to 2.0 M NaCl or KCl for those of the crude enzyme (10). The salt response pattern of the purified enzyme at pH 7.0, 50°C, resembled that of the crude enzyme at pH 5.0, 30°C (10), whereas when phosphate buffer (pH 7.0) was used instead of Tris-hydrochloride buffer (pH 7.0), the purified enzyme required 1 M NaCl or 2 M KCl for the maximal activity at 50°C. Although phosphate buffer is known to be a sequestering agent for calcium (17), the reason for such changes in halophilic properties of the enzyme could not be explained.
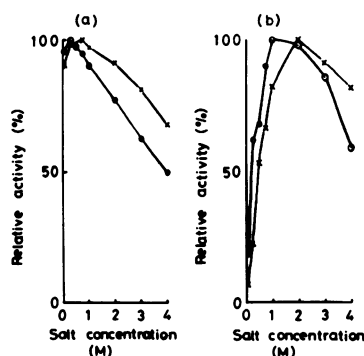

FIG. 6. *Effects of NaCl and KCl concentrations on the activity of the Micrococcus amylase. The assay was carried out at 50°C in (a) 33 mM Tris-hydrochloride buffer (pH 7.0) and (b) 33 mM phosphate buffer (pH 7.0) containing various concentrations of NaCl (⊙) or KCl (×) for 10 min. The amylase activity taken as 100% in each assay was (a) 78.6 U/ml in 0.25 M NaCl and 81.6 U/ml in 0.75 M KCl, (b) 23.2 U/ml in 1.0 M NaCl and 37.1 U/ml in 2.0 M KCl.*
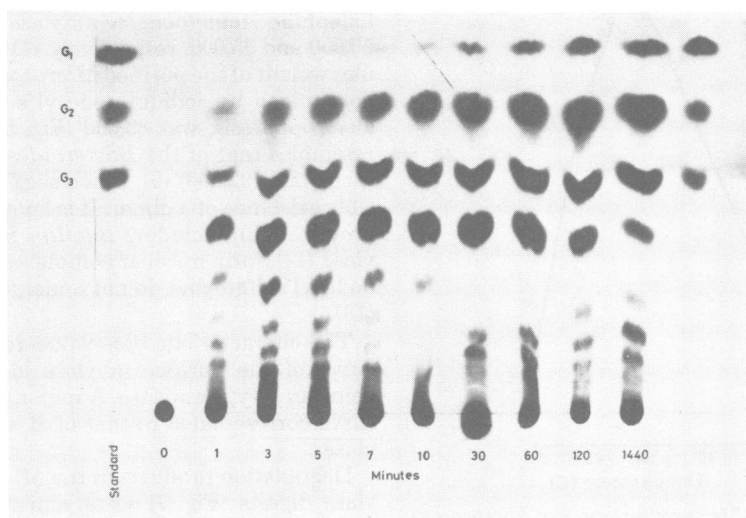
FIG. 7. *Chromatographic analysis of soluble starch digestion by the purified amylase. The reaction was carried out in 28 mM acetate buffer (pH 6.5) containing 0.143 M NaCl at 50°C for the times indicated. The samples taken at various times were applied to the paper chromatogram.*

All α-amylases studied in detail were calcium-metallo-enzymes and required calcium for optimal enzyme activity (22). The dialysis experiments indicated that the enzyme was dependent on Ca^{2+} ion for stability. Activation with added Ca^{2+} was not examined because the enzyme preparation contained 50 mM $CaCl_2$. The inactivated enzyme after ethylenediaminetetraacetic acid dialysis could not restore the activity by dialysis against 50 mM $CaCl_2$.

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